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# Multiple Heteroreceptors on Limbic Thalamic Axons: $M_2$ Acetylcholine, Serotonin$_{1B}$, $\beta_2$-Adrenoceptors, $\mu$-Opioid, and Neurotensin

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*KEY WORDS* Presynaptic receptors, Cingulate cortex, Limbic system, Serotonergic system, Autoradiography

*ABSTRACT* Ligand binding to many transmitter receptors is much higher in layer Ia of rat posterior cingulate cortex than it is in other layers, and this is where most axons from the anterior thalamus terminate. The present study explores the possibility that a number of receptors may be expressed on axons from limbic thalamic nuclei that terminate in layer Ia. Unilateral thalamic lesions were placed in rats and, 2 weeks later, five ligand binding protocols, coverslip autoradiography, and single grain counting techniques were used to quantify binding in control and ablated hemispheres. Binding to the following receptor subtypes was analyzed: $M_2$ acetylcholine, $^3$H-oxotremorine-M, or $^3$H-AF-DX 116 with 50 nM pirenzepine; serotonin$_{1B}$, $^{125}$I-(−)-cyanopindolol with 30 $\mu$M isoproterenol; $\beta_2$-adrenoceptors, $^{125}$I-(−)-cyanopindolol with 1 $\mu$M serotonin and 10 $\mu$M atenolol; $\mu$-opioid, $^3$H-Tyr-D-Ala-Gly-MePhe-Gly-ol; neurotensin, $^3$H-neurotensin. Thalamic lesions reduced binding in two laminar patterns. In one pattern, there was a major reduction in binding in most superficial layers with that in layer Ia ranging from 50 to 70% for binding to $M_2$ muscarinic and serotonin$_{1B}$ receptors. Binding to $\beta_2$-adrenoceptors was also reduced in most superficial layers but to a lesser extent. In the second pattern, reductions were limited to layer I with losses in layer Ia of 20–30% for $\mu$-opioid and neurotensin receptors. In no instance was layer Ia binding completely abolished (i.e., postlesion peaks remained).

Since the transmitters for each of the five receptors analyzed in this study are not synthesized by anterior or laterodorsal thalamic neurons, these receptors are heteroreceptors. The greatest postlesion reduction in $M_2$ binding was for AF-DX 116 and so most $M_2$ heteroreceptors are of the "cardiac" subtype. Finally, the diverse population of heteroreceptors on limbic thalamic axons provides for presynaptic modulation by a wide range of transmitter systems and suggests that thalamocortical transmission may not be a simple, unmodulated event.

## INTRODUCTION

Axonal receptors that have a high affinity for a transmitter other than that released by the axon are termed heteroreceptors. Many heteroreceptors have been characterized in the central nervous system (CNS); they are mainly involved in modulating transmitter release. Serotonin (5-HT) heteroreceptors are among the most widely distributed and have been shown to inhibit the release of acetylcholine (ACh) (Maura and Raiteri, 1986; Barnes et al., 1989), dopamine (Ennis et al., 1981), and glutamate (Raiteri et al., 1986; Bobker and Williams, 1989), while enhancing norepinephrine release (Feuerstein and Hertting, 1986). Since binding to 5-HT$_1$ receptors is reduced in the superior colliculus following retinal lesions, 5-HT receptors may also be on nonserotonergic ganglion cell axons (Segu et al., 1986).

Numerous types of heteroreceptors may be located on a single class of axon terminal, providing for the possibility of multiple transmitter interactions on the axon. For example, the release of dopamine from nigrostriatal

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terminals is depressed by acetylcholine (de Balleroche et al., 1982; Raiteri et al., 1984), 5-HT (Ennis et al., 1981) and the $GABA_B$ agonist baclofen (Bowery et al., 1980), while its release is potentiated by neurotensin (Jiang et al., 1988), δ-opioid-selective agonists (Chesselet et al., 1981), and thyrotropin-releasing hormone (Kerwin and Pycock, 1979). Autoradiographic studies have also suggested that δ-opioid receptors are on these terminals (Goodman et al., 1980; Murrin et al., 1980). Whether all these receptors are on the same axon terminal or are on different subclasses of dopaminergic terminals has not been determined.

Activity in the axons of thalamocortical projection neurons may be modulated by acetylcholine via muscarinic heteroreceptors. In situ hybridization (Buckley et al., 1988) and experimental radioligand binding (Vogt and Burns, 1988) studies have shown that neurons in the anteroventral thalamic nucleus synthesize $M_2$ acetylcholine receptors that are transported to thalamic axon terminals in layer Ia of posterior cingulate cortex. Lesion studies have also shown that most thalamic axons terminate in layer Ia of this cortex (Vogt et al., 1981). Since neurons in the anteroventral nucleus are not cholinergic; that is, they do not produce the synthetic enzyme choline acetyltransferase (Kimura et al., 1981; Armstrong et al., 1983); these $M_2$ receptors are heteroreceptors. The cerebral cortex contains $M_2$ receptors with a high or low affinity for the muscarinic antagonist AF-DX 116 and have been termed "cardiac" and "glandular" receptors, respectively (Giachetti et al., 1986; Hammer et al., 1986; Giraldo et al., 1987; Wang et al., 1987). Since AF-DX 116 blocks acetylcholine-mediated inhibition of aspartate release in the hippocampus (Raiteri et al., 1990), the "cardiac" receptors can be heteroreceptors. It is possible, therefore, that heteroreceptors on thalamic axons have a high affinity for AF-DX 116 and are also of the "cardiac" subtype.

Radioligand binding to $M_2$ heteroreceptors in posterior cingulate cortex is at highest density in layer Ia (Vogt and Burns, 1988). Over the past 10 years, a number of ligands for other receptors have been reported that also have peak densities in layer Ia and are therefore candidates for being heteroreceptors on thalamic axons as well. Receptors that are highest in density in layer I as compared with deeper layers of posterior cingulate cortex include the following: 5-$HT_{1B}$ (Crino et al., 1990), β-adrenoceptors (Rainbow et al., 1984), μ-opioid (Lewis et al., 1983; McLean et al., 1986), and neurotensin (Young and Kuhar, 1981).

In the present study, ligand binding to each of the above noted receptors was evaluated in posterior cingulate cortex following thermocoagulation or neurotoxin lesions in the limbic thalamus. Since all five classes of receptors were reduced by these lesions, it appears that thalamocortical axons that project to cingulate cortex may be under the control of multiple neurotransmitter systems. This organization provides for a much wider range of presynaptic integrative activities than has previously been known for any cortical afferent.

## MATERIALS AND METHODS

Unilateral thalamic or cortical undercut ablations were made as previously reported (Vogt et al., 1981; Vogt, 1984) in 56 hooded Long-Evans male rats (300–350 g). The animals were anesthetized with Chloropent (0.32 ml/100 g body weight, Fort Dodge Laboratories, Fort Dodge, IA) and placed in a Kopf Small Animal Stereotaxic Instrument. Angled steel electrodes were inserted through a cranial incision at two anteroposterior levels of thalamus. One mA of current (DC+) was passed for 8 sec using a constant-current lesion maker (Grass Instruments Co., Quincy, MA). Undercut lesions were made with a scalpel blade that was passed 0.9 mm lateral to the midline at a 3-mm depth through area 29d. To ensure that anterior cingulate and subicular afferents were also severed, coronally oriented knife cuts were made up to 1.5 mm from the midline and at 1.7 mm and 5.3 mm behind the bregma. In 12 cases, ibotenic acid was injected into the thalamus, to avoid destruction of axons of passage. In these cases, two 0.3-μl injections of 1 mg ibotenic acid in 100 μl 0.9% saline were made into the thalamus. These ablations were similar in extent to the thermocoagulation lesions. Following a 2-week postoperative survival period, animals were sacrificed with $CO_2$ and intracardially perfused with 50–100 ml of 15°C Krebs–Henseleit buffer. The brains were removed and frozen to −70°C in hexane. Cryostat microtome sections were cut at 16-μm thickness and mounted on chrome–alum–coated slides. A total of 2,400 slides with 2–10 sections per slide were prepared and analyzed for these studies.

Nissl-stained sections through the thalamus were reconstructed with an Aus Jena projector, a representative example of which is shown in Figure 1. In these cases, the anterior nuclei were either completely destroyed and/or their efferent axons severed en route to the cerebral cortex. This was also true for the midline paraventricular, parataenial, and reuniens nuclei. At caudal levels of the thalamus, the laterodorsal, centrolateral, and most of the mediodorsal nuclei were destroyed. The posterior and ventroposterior nuclei were not consistently involved in these lesions.

Pirenzepine was kindly provided by Boehringer Ingelheim, Ltd., zinterol by Bristol Myers Company, and levallorphan by Hoffmann La Roche, Inc. Serotonin, atropine, and atenolol were purchased from Sigma Chemical Company (St. Louis, MO), and isoproterenol was purchased from Research Biochemicals, Inc. (Natick, MA). Radiolabeled ligands were purchased from New England Nuclear (Boston, MA) and included the following: $^{3}$H-oxotremorine-M (OXO-M, sp. act. 85.1 Ci/mM); $^{3}$H-AF-DX 116 (sp. act. 59.8 Ci/mM); $^{125}$I-(−)-cyanopindolol (CYP, sp. act. 2,200 Ci/mM); $^{3}$H-Tyr-D-

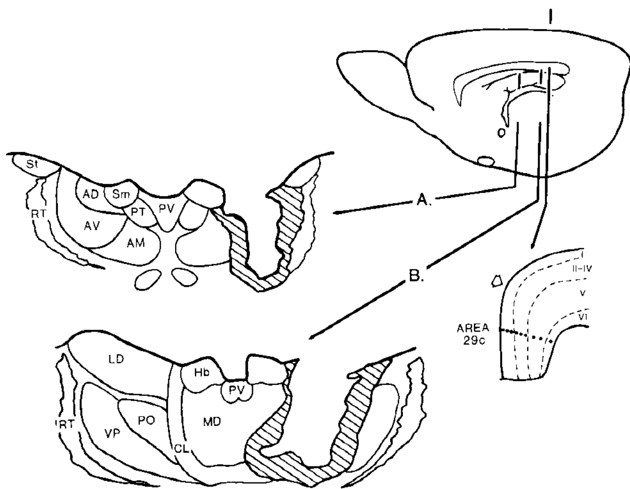


Fig. 1. Medial surface of the rat brain shown with two levels of the thalamus from a representative thermocoagulation lesion case. Necrotic tissue is shown with cross-hatching. A level of area 29c is also indicated as are the nine layers in which specific ligand binding was quantified. Thalamic nuclei and straie: AD, anterodorsal; AM, anteromedial; AV, anteroventral; CL, centrolateral; Hb, habenula; LD, laterodorsal; MD, mediodorsal; PO, posterior; PT, parataenial; PV, paraventricular; RT, reticular; Sm, stria medullaris; St, stria terminalis; VP, ventroposterior.

Ala-Gly-MePhe-Gly-ol (DAGO, sp. act. 30.3 Ci/mM); $^{3}$H-neurotensin (sp. act. 80.7 Ci/mM).

The conditions for ligand binding experiments have been previously reported (Young and Kuhar, 1981; Plager and Vogt, 1988; Slesinger et al., 1988; Vogt and Burns, 1988; Crino et al., 1990). All buffers were at pH 7.4:

*Oxotremorine-M:* Sections were incubated in 0.1 nM oxotremorine-M in 20 mM HEPES–Tris buffer with 10 mM magnesium and 50 nM unlabeled pirenzepine (OXO-M/PZ) for 30 min at 25°C, followed by two buffer washes at 4°C for 2 min each and one 2-min water wash. Nonspecific binding was assessed with 1 μM atropine in a parallel series of sections.

*AF-DX 116:* Sections were preincubated in a 50-mM sodium–potassium phosphate buffer for 30 min at 25°C, then incubated in 5 nM $^{3}$H-AF-DX-116 in the same buffer for 30 min at 25°C, followed by a 3-min buffer wash at 4°C and a water wash for 1 min at 4°C. Nonspecific binding was evaluated with 1 μM atropine in a parallel series of sections.

*Cyanopindolol:* Conditions for cyanopindolol binding to the serotonin$_{1B}$ receptor were determined by Hoyer et al. (1985) and Offord et al. (1988), and our autoradiographic technique follows that of Pazos et al. (1985). Sections were incubated in 20 pM $^{125}$I-(−)-cyanopindolol with 30 μM unlabeled isoproterenol to block β-adrenoceptor binding (CYP/IPT) for 120 min at 25°C in covered containers to minimize evaporation following a 10-min preincubation at 25°C in 0.17 M Tris buffer with 150 mM sodium chloride. Following incubation in the iodinated ligand, the sections were washed two times in buffer at 4°C for 5 min each. Nonspecific binding was determined with 1 μM serotonin in a parallel series of sections.

*Cyanopindolol:* Binding of cyanopindolol to β-adrenoceptors and not serotonin receptors is ensured in this assay by including serotonin in the incubation medium. Thus, sections are incubated in 20 mM Tris buffer with 135 mM sodium chloride and 65 pM $^{125}$I-(−)-cyanopindolol with 1 μM serotonin (CYP/5-HT) for 120 min at 25°C in covered containers. The sections were then washed three times in buffer at 4°C for 20 min each and one time in water for 5 sec at 4°C. Nonspecific binding was evaluated in a parallel series of sections with 1 μM (−)-propranolol. In order to subtype the β-adrenoceptor binding either 26 nM zinterol or 10 μM atenolol was included in the incubation medium.

*Tyr-D-Ala-Gly-MePhe-Gly-ol:* Sections were incubated in 50 mM Tris with 1 nM $^{3}$H-DAGO at 25°C for 45 min, followed by three buffer washes at 4°C for 1 min each. Nonspecific binding was evaluated in a parallel series of sections with 1 μM levallorphan.

*Neurotensin:* Sections were incubated in 0.17 M Tris with 0.05% bovine serum albumin (BSA), 0.1 nM bacitracin, and 4 nM $^{3}$H-neurotensin at 25°C for 45 min. The sections were then washed in buffer at 4°C for 5 min and 2 times in water at 4°C for 5 min each. Nonspecific binding was determined with 2 μM neurotensin in a parallel series of sections.

Since one of the main issues raised in these studies is the association of multiple heteroreceptors with thalamic afferents, all cases were processed for binding to three or more receptors. Thus, OXO-M/PZ binding was assessed in most cases as an adjunct to confirmation of the lesion site in the Nissl-stained thalami. Specific binding of oxotremorine-M/PZ, CYP/IPT, DAGO, and neurotensin was evaluated in each of 16 cases with thalamic lesions. Other combinations of ligand binding protocols, including that for AF-DX 116, were performed in other experiments to confirm the findings of these cases.

Autoradiographs were prepared using the coverslip technique of Young and Kuhar (1979) as previously

reported (Vogt and Burns, 1988). Coverslips were acid-cleaned and dipped in Kodak NTB-2 emulsion. The dried coverslips were attached to slides with cyanoacrylate and exposed in the dark at −20°C for 3 weeks to 4 months. All autoradiographs were developed in Kodak D-19 without hardener, fixed in Kodak Rapid Fixer, and then counterstained with thionin.

The cytoarchitecture and connections of rat posterior cingulate cortex have been described (Vogt and Peters, 1981; Vogt, 1985), and the part of area 29c that was analyzed in this study is shown in Figure 1. In the present study, layer II–III of area 29c was subdivided into separate layers II and III as suggested by Sripanidkulchai and Wyss (1987). Each layer in these autoradiographed sections was first identified in thionin-stained sections with bright-field optics; dark-field illumination was then used so that individual grains in the overlying autoradiograph could be analyzed with a computerized image analysis system (Image Technology, model 1000, Donsanto Corp., Natick, MA). Grains were counted per 2,500 $\mu m^2$ of nine cortical layers (Fig. 1) in three nonadjacent sections incubated with or without a blocker. The machine readings were visually corrected for miscounts caused by overlapping grains and then nonspecific binding subtracted from total binding, to determine specific binding. The thickness of most layers was unaltered in the ablated hemisphere; however, there was an overall shrinkage in the thickness of layer I; 165 ± 4 μm thickness in control vs. 137 ± 3 μm thickness in ablated hemispheres. In order to account for this, the binding in each subdivision of layer I in the ablated hemispheres was reduced by 5%. While increasing the accuracy of postlesion changes in receptor binding, this correction did not alter the overall conclusions. Mean values ±SEM were calculated for each layer for control and ablated hemispheres. Only two exactly matched groups were used for each analysis. Differences between the means for each layer in experimental and control contralateral hemispheres were analyzed with Student's t-test and differences associated with $P < 0.05$ accepted as significant. These differences are indicated with stars in Figures 2, 3, 5, and 6.

It is theoretically possible that thalamic deafferentation lesions could alter postsynaptic receptor densities (e.g., Hattori and Fibiger, 1982; McKinney and Coyle, 1982). However, it is unlikely that post-thalamic lesion changes in binding in the present study are due to such transsynaptic events for the following reasons. First, electron microscopic studies 2 or 3 days following thalamic lesions, when terminal degeneration is maximal, failed to show evidence of transneuronal degeneration (Vogt et al., 1981). Second, high levels of acetylcholinesterase activity is present in anteroventral neurons, and postlesion activity of this enzyme decreases in layer Ia at a rate that approximates changes in muscarinic receptor binding (Vogt, 1984). Third, in cases in which almost all dendrites have been removed with ibotenic acid lesions, peaks in ligand binding remain in layers Ia and III/IV. This is true for muscarinic (Vogt, 1984, 1985; Vogt and Burns, 1988) and serotonin (Crino et al., 1990) receptor antagonists, which are likely associated with afferent axons. Fourth, receptors that have a dendritic localization based on post-ibotenic acid cortical neuron degeneration are not altered by thalamic lesions or undercut lesions that remove all inputs to cortex. This is true for pirenzepine (Vogt and Burns, 1988) and 8-hydroxy-2-(di-n-propylamino)tetralin binding (Crino et al., 1990). Finally, in situ hybridization studies (Buckley et al., 1988) have shown that $M_2$ receptors are synthesized in the anteroventral thalamic nucleus.

## RESULTS

### $M_2$ acetylcholine receptor binding

Specific binding of 0.1 nM $^3$H-oxotremorine-M coincubated with 50 nM unlabeled pirenzepine (OXO-M/PZ) was highest in layer Ia, moderate in layer III, and low in the remaining layers as shown in Figure 2. Binding of OXO-M/PZ was significantly reduced in layers I-III of area 29c following thalamic lesions. The mean values for 13 cases are plotted in Figure 2. In all experiments, the average loss in layer Ia was 50–60%.

Specific $^3$H-AF-DX 116 binding was highest in layer Ia, while it was at moderate levels in layers Ib-Vb and low in layer VI as shown in Figure 3. Following thalamic lesions, AF-DX 116 binding was reduced in layers Ia, Ib, II, and Vb. Inclusion of 50 nM unlabeled pirenzepine with the $^3$H-AF-DX 116 resulted in reduced binding in all layers (AF-DX 116/PZ) of control cortices but left high levels of binding in layer Ia. Since the autoradiographs for Figure 3 were prepared from the same cases and developed for the same time and under the same conditions, quantitative differences in binding between Figures 3A and 3B are of interest. Thalamic lesions reduced layer Ia $^3$H-AF-DX 116 binding by 38%, while they reduced binding of AF-DX 116/PZ by 67%. A photograph of this altered binding is shown in Figure 4. This was the greatest postoperative reduction in binding observed for all ligands analyzed in the present study.

### Serotonin$_{1B}$ receptor binding

The laminar distribution of $^{125}$I-(−)-cyanopindolol binding with 30 μM unlabeled isoproterenol (CYP/IPT) is shown in Figure 2. In control hemispheres, highest levels of CYP/IPT binding were in layer I, while there was moderate binding in layers II and III and low binding in deeper layers. Thalamic thermocoagulation lesions reduced CYP/IPT binding in layers I–III (see also Fig. 4). Binding in layers Ia and Ib was reduced by the greatest amount of 52% and 41%, respectively, and a postoperative peak remained in layer I. Since it has been shown that CYP/IPT binds to presynaptic terminals that originate in the raphe nuclei (Crino et al., 1990), axons of passage from these and other brainstem nuclei could be damaged by thermocoagulation lesions

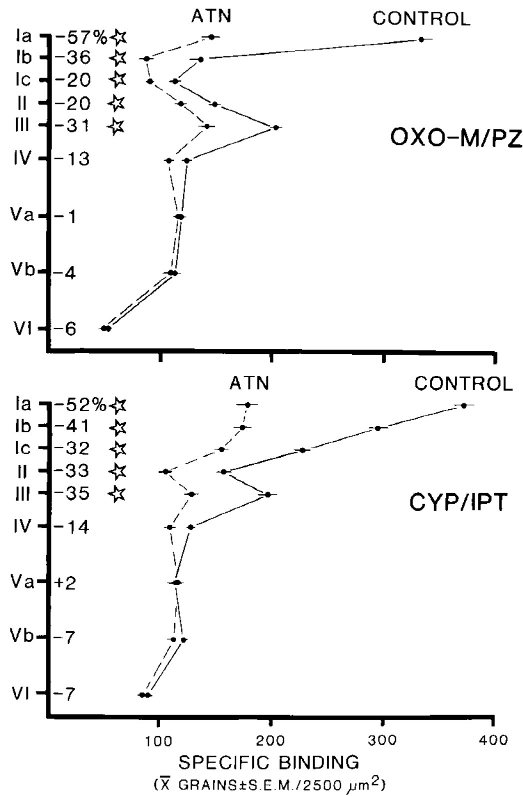


Fig. 2. Laminar distributions of OXO-M/PZ and CYP/IPT binding in control and thalamic ablated (ATN) hemispheres from 13 and 18 cases, respectively. The percentage reduction in binding following the lesions is shown to the left with stars next to those values that are associated with significantly different means.

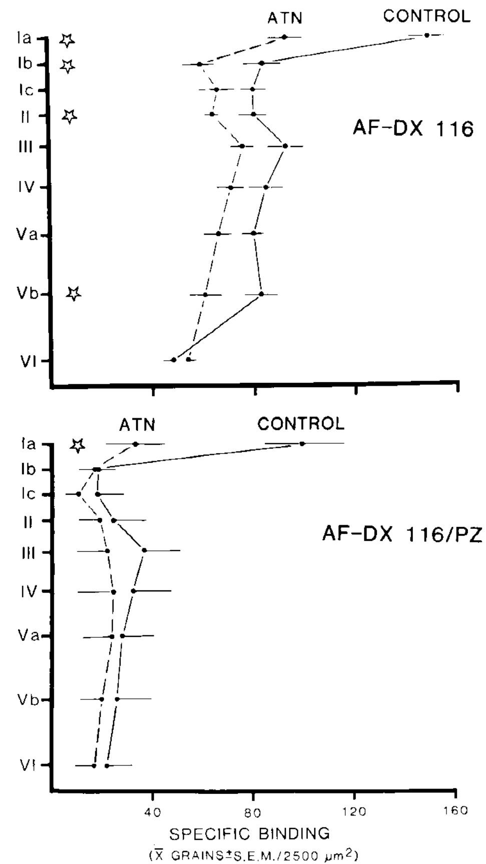


Fig. 3. Binding of $^3$H-AF-DX 116 without (top) or with (bottom) 50 nM pirenzepine in 6 cases that had unilateral thalamic lesions (ATN). The stars to the left are associated with mean values that were significantly different.

in the thalamus. Therefore, ibotenic acid was injected into the thalamus, to avoid axons of passage. Binding of CYP/IPT in these cases was reduced in layers I–III to exactly the same extent as noted for the cases with thermocoagulation lesions.

### β-Adrenoceptor binding

The laminar profile of $^{125}$I-(−)-cyanopindolol binding co-incubated with 1 μM serotonin (CYP/5-HT) is presented in Figure 5. Specific CYP/5-HT binding was highest in layer I and moderate in all deeper layers. Ibotenic acid or thermocoagulation lesions in the thalamus reduced CYP/5-HT binding in layers Ia, Ib, and III (see also Fig. 4). Inclusion of 26 nM zinterol in the incubation buffer to block $\beta_2$-adrenoceptor binding amplified the proportion of binding in layers Ic, IV, and V in control hemispheres (Fig. 5). The binding of CYP/5-HT under these conditions was unaltered following thalamic lesions. By contrast, inclusion of 10 μM of the $\beta_1$-antagonist atenolol in the incubation buffer maintained the normal pattern of CYP/5-HT binding with highest levels in layer I. Under these conditions, a significant reduction in CYP/5-HT binding was uncovered in layers Ia, Ib, Ic, and III of 25%, 27%, 24%, and 28%, respectively. Thus, it was likely that a component of CYP/5-HT binding to $\beta_2$-adrenoceptors was on thalamic axon terminals.

### μ-Opioid receptor binding

Specific binding of DAGO had two broad peaks in area 29c of control hemispheres, one in layer I and the other in layers IV and Va, as shown in Figure 6. Cortical undercut lesions that essentially destroyed all afferent axons to area 29c significantly reduced DAGO binding in layers Ia, Ib, and Ic by 63%, 64%, and 53%, respectively (data not shown). Binding was not altered in any of the deeper layers. Figure 6 shows 11 cases with thalamic lesions in which there were significant reductions in specific DAGO binding of 21%, 24%, and 18% in layers Ia, Ib, and Ic, respectively. Binding in cases with ibotenic acid or thermocoagulation lesions was combined because the lesion effects were the same. Thus, a component of DAGO binding was associated with tha-

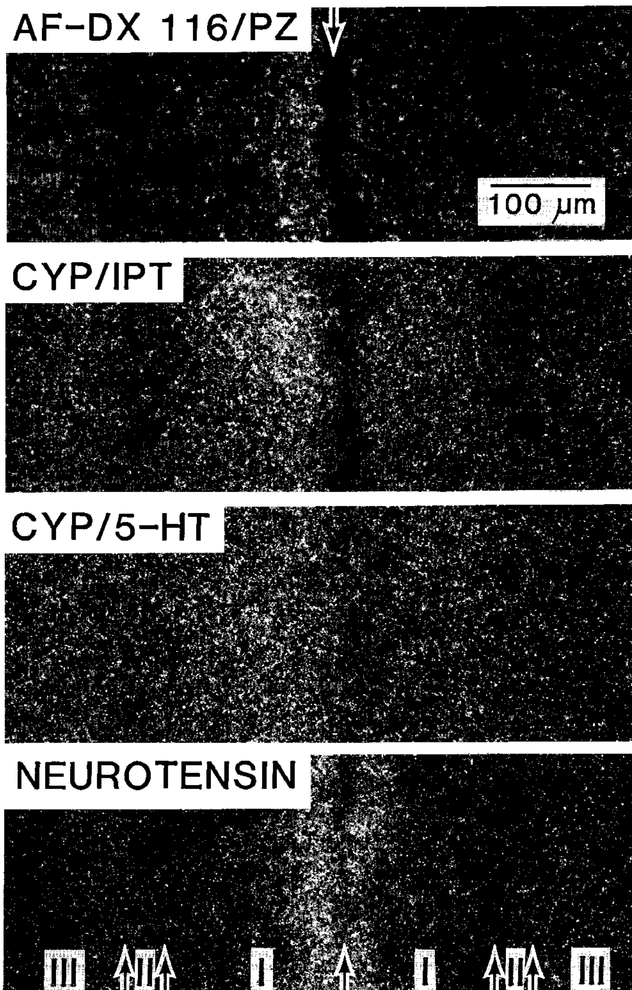


Fig. 4. Dark-field photomicrogrpahs of autoradiographs of ligand binding in superficial layers of area 29c in control (**left**) and thalamic ablated (**right**) hemispheres. The two arrows at the middle, top, and bottom mark the interhemispheric space. The cortical layers are delineated along the bottom.

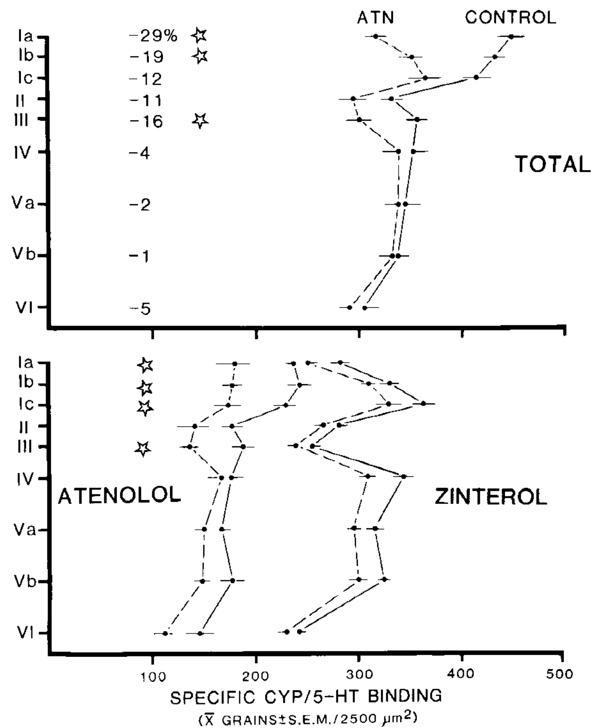


Fig. 5. Laminar distribution of total CYP/5-HT binding (**top**) and that which was unblocked with either atenolol or zinterol (**bottom**). Although there were no postlesion changes (ATN) in the zinterol-blocked series, lesion-induced changes in total CYP/5-HT binding were increased in the atenolol-blocked series. The means are for 12, 8, and 13 cases of total, atenolol, and zinterol, respectively.

lamic axons, but this did not account for all presynaptic binding sites in layer I.

## Neurotensin receptor binding

Figure 6 shows that the normal distribution of neurotensin binding in area 29c was composed of three densities. There was very high binding in layer Ia, moderate binding in layers Ib–III, and low levels in layers IV–VI. Ibotenic acid and thermocoagulation lesions in the anterior thalamus reduced neurotensin binding only in layer Ia and that was by 30% (see also Fig. 4).

# DISCUSSION

This is the first report that binding to five receptor subtypes can be altered with the removal of a single cortical input (i.e., thalamocortical afferents). Neurons in the limbic thalamus including those in the anterior and laterodorsal nuclei are not known to synthesize the ligands for these receptors, so most or all of these receptors are probably heteroreceptors. Furthermore, we have been unable to find axo-axonal synapses in layer Ia of rat posterior cingulate cortex using electron microscopic techniques or in cortex, where most dendrites were removed with ibotenic acid (unpublished observations). Therefore, these heteroreceptors are likely stimulated by transmitters from distant axons. Thus, limbic thalamic transmission to cortex may not be a simple, unmodulated event; rather, it may be regulated by numerous transmitters via nonsynaptic heteroreceptor interactions.

## $M_2$ acetylcholine receptors

There is in situ and experimental radioligand binding evidence that $M_2$ acetylcholine receptors are produced by neurons in the anteroventral thalamic nucleus (Vogt and Burns, 1988; Buckley et al., 1988). Two types of $M_2$ acetylcholine receptors have been identified in the cerebral cortex. The "cardiac" type has a high affinity for the muscarinic antagonist AF-DX 116, comprises 15% of the cortical muscarinic receptor population and modulates the release of aspartate, while the "glandular" or $M_3$ type has a low affinity for AF-DX 116 and comprises

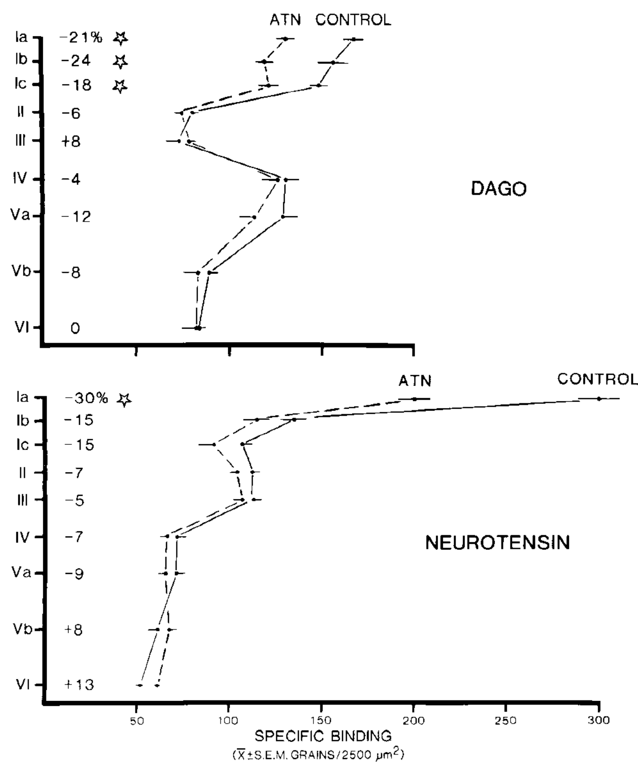


Fig. 6. Laminar distribution of specific binding for DAGO and neurotensin in 11 cases that had unilateral thalamic lesions (ATN). Binding of DAGO was reduced in all subdivisions of layer I, while that for neurotensin was only reduced in layer Ia.

35% of cortical muscarinic sites (Giachetti et al., 1986; Hammer et al., 1986; Giraldo et al., 1987; Wang et al., 1987; Raiteri et al., 1990). The present study shows that $M_2$ heteroreceptors on anterior thalamic axons are predominantly of the "cardiac" type. Thus, when compared to OXO-M/PZ binding, thalamic lesions produced greater overall losses in AF-DX 116/PZ binding in layer Ia of 67%. In addition, since pirenzepine does not bind to thalamic axons (Vogt and Burns, 1988) and co-incubation of pirenzepine with $^3$H-AF-DX 116 enhanced the reduction in binding following thalamic lesions, it appears that at a 5 nM concentration of AF-DX 116, there was significant binding of AF-DX 116 to pirenzepine-sensitive sites in cingulate cortex.

### Serotonin$_{1B}$ receptors

Postlesion reductions in CYP/IPT binding were substantial in most superficial layers of posterior cingulate cortex suggesting that limbic thalamic neurons also synthesize large numbers of 5-HT$_{1B}$ receptors. Since neurons in the thalamus are not immunoreactive for serotonin (Cropper et al., 1984), they are probably not serotonergic. Thus, 5-HT$_{1B}$ receptors are heteroreceptors on thalamocortical axons. In light of the predominant role of serotonin in inhibiting transmitter release via 5-HT$_{1B}$ heteroreceptors (Maura and Raiteri, 1986; Raiteri et al., 1986; Bobker and Williams, 1989), it is possible that the 5-HT$_{1B}$ receptors on thalamic axons inhibit transmitter release.

Serotonin has been implicated in mammalian learning. Ögren (1985) and Ögren and Johansson (1985) have shown that it is involved in the acquisition of active avoidance tasks. Removal of cingulate cortex itself also interferes with active avoidance learning (Peretz, 1969; Thomas and Slotnick, 1963; Lubar, 1964). We propose that one of the key sites where active avoidance learning may be established is at the 5-HT$_{1B}$ heteroreceptors on anterior thalamic axon terminals.

### $\beta_2$-Adrenoceptors

$\beta$-adrenoceptors are at peak densities in layers I and II in the cerebral cortex and are likely both postsynaptic on cortical neurons and presynaptic on adrenergic afferents from the locus coeruleus, i.e., autoreceptors (Palachios and Kuhar, 1980; Levin and Biegon, 1984; Rainbow et al., 1984). In a report by Cash et al. (1986), it was suggested that "beta$_2$ receptors may be situated on glial cells or neuronal elements unrelated to noradrenergic input." The present study provides evidence for a specific location for these latter receptors on thalamocortical projection neurons. Reduced binding to $\beta_2$-adrenoceptors following thalamic lesions could also be the result of changes in the expression of these receptors by glial cells. Nahorski et al. (1979) suggested that $\beta_2$-adrenoceptors were expressed by glial cells because this binding survived neuron removal with kainic acid. Since layer I of cingulate cortex has a high number of glial cells (Aoki et al., 1987), it is possible that during phagocytosis of thalamic terminals these cells temporarily stop expressing $\beta_2$-adrenoceptors.

It has been proposed that the joint actions of noradrenaline and acetylcholine are responsible for neuronal plasticities in the developing cerebral cortex (Bear and Singer, 1986; Greuel et al., 1988). Although the joint actions of these transmitters during adult learning are not known, the thalamocortical axon terminal is likely a critical site for interactions between these two transmitter systems.

### $\mu$-Opioid receptors

The presence of opioid receptors on thalamic terminals in cingulate cortex was hypothesized by Lewis et al. (1983) and McLean et al. (1986) on the basis of the laminar codistribution of $\mu$-opioid receptors and thalamic axon terminals. Experimental observations of the present study confirmed this suggestion. It is interesting to note, however, that reductions in binding to mu opioid and neurotensin receptors was limited to layer I. This could mean that these receptors are produced by a division of the anterior nucleus other than the anteroventral division or the laterodorsal nucleus. Finally, since stimulation of opioid heteroreceptors reduces

norepinephrine release (Schoffelmeer and Mulder, 1982; Jackisch et al., 1986), some heteroreceptors on anterior thalamic axons, including $\mu$-opioid sites, may reduce transmission through the thalamocortical projection.

### Neurotensin receptors

Of the five transmitter systems considered in the present study, only neurotensin may be synthesized by anterior thalamic neurons. Sugimoto et al. (1985) reported neurotensin-, vasoactive intestinal polypeptide-, and cholecystokinin-like immunoreactivity in the anterior thalamic nucleus. If these neurons synthesize and release neurotensin, many of the receptors in layer Ia that are eliminated following thalamic lesions are autoreceptors. However, in a review by Emson et al. (1985), there was no evidence of neurotensin-like immunoreactivity in the rat thalamus, nor have Abrams et al. (1985) or Vanderhaeghen (1985) observed vasoactive intestinal polypeptide-like or cholecystokinin-like immunoreactivity in rat anterior thalamus. Thus, it is possible that some or all of the layer Ia neurotensin receptors are heteroreceptors.

Since the subiculum contains neurotensinergic neurons (Roberts et al., 1984; Kiyama et al., 1986) that project to layers II–III of posterior cingulate cortex (Meibach and Siegel, 1977; Finch et al., 1984), the subiculum may be a source of input for these receptors. Interestingly, it is in these layers that neurotensin binding is at moderate levels but is unaltered by thalamic lesions. Neurotensin binding is highest in layer Ia (Young and Kuhar, 1981); it is in this layer that thalamic lesions reduce binding of neurotensin (i.e., distal to subicular input). A more direct source of neurotensin for layer Ia heteroreceptors might originate from the raphe nuclei. There is a precedent for co-localization of 5-HT and neurotensin in the same neurons. Thus, the raphe nuclei receive periaqueductal gray and paragigantocellular nucleus inputs from neurons containing both 5-HT and neurotensin (Beitz, 1982). The raphe nuclei themselves project extensively to layer I of posterior cingulate cortex (Moore et al., 1978), where there are also high densities of serotonergic axons (Lidov et al., 1980) and serotonin autoreceptors (Crino et al., 1990). Since some neurons in the raphe nuclei are neurotensinergic (Beitz, 1982; Emson et al., 1985), activation of these cells could result in the release of 5-HT and neurotensin in layer Ia.

### Multiple heteroreceptors

Although it is likely that $M_2$ acetylcholine heteroreceptors are on the axons of anteroventral thalamic neurons, this need not be the case for all the heteroreceptors analyzed in this study. Sripanidkulchai and Wyss (1986) showed that neurons in the following thalamic nuclei of the rat have prominent projections to area 29c: anterodorsal, anteroventral, anteromedial, parataenial, centromedial, reuniens, rhomboid, laterodorsal, and centrolateral. Since postlesion reductions in ligand binding occurred in two different laminar patterns, it is possible that axons from different thalamic nuclei are regulated by separate populations of heteroreceptors. For example, although the anteroventral nucleus projects prominently to layer Ia (Sripanidkulchai and Wyss, 1987), projections of the anterodorsal nucleus are mostly to layer III (T. Van Groen and J.M. Wyss, personal communication). Thus, $M_2$, 5-$HT_{1B}$, and $\beta_2$-adrenoceptors may be on anteroventral and anterodorsal terminals. By contrast, terminals of laterodorsal neurons are evenly distributed throughout layer I and have only weak projections to deeper layers. Therefore, the $\mu$-opioid and neurotensin receptors could be on afferent axons of the laterodorsal thalamic nuclei.

## CONCLUSION

As many as five classes of heteroreceptors have been localized to limbic thalamic axons that terminate in layer Ia of posterior cingulate cortex. These receptors could provide for presynaptic modulation of thalamocortical axons by the cholinergic, serotonergic, adrenergic, opioidergic, and neurotensinergic systems. It is possible that afferent axons from a number of thalamic nuclei are differently regulated by these heteroreceptors.

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